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Mass spectrometric evidence for the anomalous chemical behavior of 11-dehydrothromboxane B_2

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Abstract

When we subjected 11-dehydrothromboxane B_2 (11-DTXB$_2$), a metabolite of arachidonic acid, to standard chemical derivatization procedures we obtained a mixture of several products. Separation of the components was carried out by gas chromatography and their identification was accomplished through the study of their mass spectra, which are presented here. Anomalous behaviors include methylation of allylic and alcoholic hydroxyl groups by diazomethane, unusually slow derivatization of one of the hydroxyl groups with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and etherification of another with ethanol. The underlying causes of these abnormal behaviors are not obvious, but appear to be related, at least in part, to the opening/closure of a lactone ring in the molecule. These observations have some bearing on the development of valid procedures for GC-MS quantification of this important marker of thromboxane A_2 synthesis in vivo, and of similar compounds.

Keywords: 11-dehydrothromboxane B_2; Thromboxane metabolite; Chemical behavior; Anomalous derivatizations; Electron ionization; Mass spectrometry

1. Introduction

Thromboxane A_2 (TXA$_2$) is a member of the eicosanoid family of compounds biochemically derived in mammals from arachidonic acid, a 20-carbon polyunsaturated essential fatty acid [1]. TXA$_2$ enhances the atherothrombotic process by inducing platelet aggregation and vasoconstriction [2]. Because the level of endogenous synthesis of TXA$_2$ can be altered by pharmacological and nutritional interventions, as well as by pathophysiological conditions, there is an interest in non-invasive procedures that allow monitoring of TXA$_2$ synthesis in vivo. One such procedure is the measurement, by gas chromatography-mass spectrometry (GC-MS), of the urinary excretion of 11-dehydrothromboxane B_2 (11-DTXB$_2$), a product of hydrolysis plus enzymatic transformation of TXA$_2$ and an index of endogenous TXA$_2$ production in the human circulation [3].

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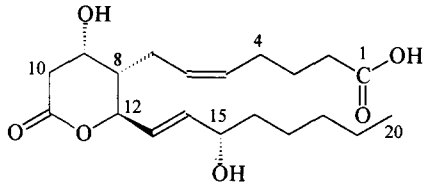


Fig. 1. 11-Dehydrothromboxane B_2 (11-DTXB_2).

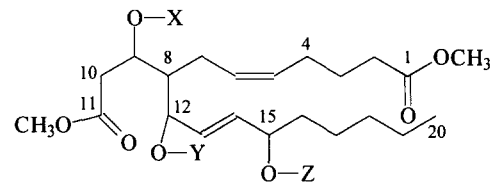
During the development of our GC-MS assay for 11-DTXB_2 [4], we observed several instances of abnormal chemical behavior of the analyte. The purpose of this paper is to report the structures of the unanticipated by-products, together with supporting mass spectrometric evidence. Structural identification of all chemical species, the mass assignments and the fragmentation pathways that we propose are based on substituent shifts, on the use of two ^{13}C-labeled analogs and on the formation of two *n*-butylboronate ester derivatives. A detailed mechanistic study of the by-products' formation is beyond the scope of this work. However, the probability that these, or related compounds, could be encountered by other analysts prompted us to report their identification and the data supporting their structural assignments.

2. Results

When we subjected 11-dehydrothromboxane B_2 (Fig. 1) to the sequence of treatments shown in Scheme 1, to evaluate the length of time required for the closure of the lactone ring, we obtained a mixture of several derivatives. The structures of the compounds identified are depicted in Figs. 2 and 3.

2.1. Mass spectral characterization of the identified compounds

The electron ionization mass spectra of **1–7** are presented in Tables 1 and 2. The spectrum of **8**, with a molecular ion of *m/z* 468 (< 1%), displays a base peak at *m/z* 397 originating from the loss of $^{\cdot}C_5H_{11}$. Elimination of $^{\cdot}CH_3$ and CH_3OH from $M^{+\cdot}$ results in fragments at *m/z* 453 (2%) and *m/z* 436 (1%), respectively. Evidence of neutral plus radical losses is found at *m/z* 405 (4%), $[M-(CH_3OH + CH_3O^{\cdot})]^+$, *m/z* 365 (85%), $[M-(CH_3OH + {}^{\cdot}C_5H_{11})]^+$ and *m/z* 275 (64%), $[M-(TMSOH + CH_3OH + {}^{\cdot}C_5H_{11})]^+$. Other significant fragments are observed at *m/z* 141 (25%) from cleavage at C-7/C-8, *m/z* 173 (26%) from cleavage at C-14/C-15, and *m/z* 199 (27%) from fission at C-12/C-13. The origin of a relatively abundant ion (41%) at *m/z* 243 is uncertain. The spectrum of **9** was described previously [5].

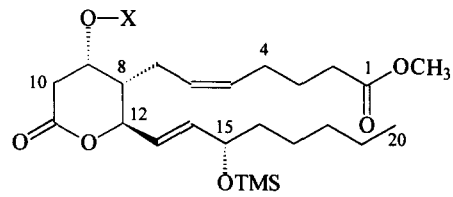


	X	Y	Z
1	TMS	CH_3	TMS
2	TMS	TMS	CH_3
3	CH_3	TMS	TMS
4	TMS	CH_3	CH_3
5	CH_3	CH_3	CH_3
6	TMS	TMS	TMS
7	CH_3	TMS	CH_3

Fig. 2. Products identified in the derivatization mixture (open form).

2.2. Artifacts

The ethyl ether homolog of **1** at C-12 was also identified. Residual ethanol used to azeotropically



	X
8	CH_3
9	TMS

Fig. 3. Products identified in the derivatization mixture (lactones).

Table 1
EI mass spectra of derivatives **1**–**4** of 11-dehydrothromboxane B_2 (relative abundance in parentheses)

Ion assignment	Compound-(*m/z*)			
	1	**2**	**3**	**4**
$[M]^{+\cdot}$	572[a]	572 (<1)	572 (<1)	514 (<1)
$[M-{}^{\cdot}CH_3]^+$		557 (<1)	557 (<1)	
$[M-CH_3O^{\cdot}]^+$	541 (<1)	541 (<1)	541 (<1)	483 (2)
$[M-CH_3OH]^{+\cdot}$			540 (<1)	482 (5)
$[M-(CH_3OH + {}^{\cdot}CH_3)]^+$	525 (3)	525 (2)	525 (<1)	467 (3)
$[M-(CH_3OH + CH_2O^{\cdot})]^+$			509 (1)	451 (2)
$[M-(2XCH_3OH)]^+$				450 (3)
$[M-{}^{\cdot}C_5H_{11}]^+$		501 (<1)		
$[M-TMSOH]^{+\cdot}$	482 (3)	482 (3)	482 (3)	424 (3)
$[M-(CH_3OH + {}^{\cdot}C_5H_{11})]^+$				411 (26)
$[M-(TMSOH + CH_3O^{\cdot})]^+$	451 (5)			
$[M-(TMSOH + CH_3OH)^{\cdot}]^+$	450 (4)	450 (8)	450 (3)	
$[M-(TMSOH + {}^{\cdot}C_5H_{11})]^+$	411 (8)	411 (6)	411 (3)	
$[M-\{TMSOH + {}^{\cdot}(ZO-CH-C_5H_{11})\}]^+$				309 (15)
$[YO-CH-CH=CH-CH(OZ)-C_5H_{11}]^+$ (*a*)	243 (100)	243 (100)	301 (35)	185 (100)
$[HC=CH-CH(OZ)-C_5H_{11}]^+$	199 (9)	141[b] (2)	199 (4)	141[b] (9)
$[a-CH_3OH]^+$	211 (40)	211 (44)		153 (45)
$[a-TMSOH]^+$			211 (100)	
$[XO-CH-CH_2-COOCH_3]^+$	175 (71)	175 (57)	117 (7)	175 (35)
$[ZO-CH-C_5H_{11}]^+$	173 (74)	115 (16)	173 (14)	115 (28)
$[M-(TMSOH + CH_3OH + CH_3O^{\cdot})]^+$		419 (4)	419 (15)	361 (5)
$[M-\{TMSOH + (2XCH_3OH)\}]^{+\cdot}$				360 (6)
$[{}^{\cdot}CH_2-CH=CH-(CH_2)_3-COOCH_3]^+$	141 (3)	141[c] (2)	141 (1)	141[c] (9)

[a]Not observed.
[b]The *m/z* 141 fragment is also contributed by $[CH_2-CH=CH-(CH_2)_3-COOCH_3]^+$.
[c]The *m/z* 141 fragment is also contributed by $[HC=CH-CH(OZ)-C_5H_{11}]^+$.

remove moisture left over from one of the derivatization steps (e.g. methylation) could have been the supplier of the ethyl moiety. Alternatively, this could have been introduced by transesterification of 11-DTXB_2 (lactone form) with EtOH, which was originally used to prepare a stock solution. In the mass spectrum of the ethyl homolog of **1**, the molecular ion at *m/z* 586 was not observed. However, the identity of this compound could be inferred by the presence of significant ions at *m/z* 571 $[M-{}^{\cdot}CH_3]^-$, *m/z* 555 $[M-{}^{\cdot}OCH_3]^+$, *m/z* 525 $[M-({}^{\cdot}CH_3 + C_2H_5OH)]^+$, *m/z* 515 $[M-\{{}^{\cdot}CH_2-(CH_2)_3-CH_3\}]^+$, *m/z* 496 $[M-TMSOH]^{+\cdot}$, *m/z* 465 $[M-({}^{\cdot}OCH_3 + TMSOH)]^+$, *m/z* 451 $[M-(C_2H_5O^{\cdot} + TMSOH)]^+$, and *m/z* 450 $[M-(C_2H_5OH + TMSOH)]^{+\cdot}$. The base peak is at *m/z* 257 corresponding to fission of the C-8/C-12 bond with charge retention on the C-12/20 chain. This fragment then eliminates the elements of ethyl alcohol to give a relatively abundant ion of *m/z* 211. It seems reasonable to expect that the isomeric compound with the ethyloxy group at C-15 also formed, although its presence could not be confirmed.

When the hydroxyl group at C-9 failed to react with both diazomethane and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), the substrate underwent dehydration in the injection port with formation of a double bond at C-9/10. A $\Delta^{9,10}$ unsaturated monomethyl ether (at C-15) and a $\Delta^{9,10}$ unsaturated dimethyl ether derivative were identified. Predictably, they both eluted ahead of all the compounds that retained the oxygenated function at C-9. The spectrum of the former, with a TMSO function at C-12 and a $M^{+\cdot}$ at 482, showed the characteristic fragments $[M-{}^{\cdot}CH_3]^+$,

Table 2
EI mass spectra of derivatives **5**–**7** of 11-dehydrothromboxane B_2 (relative abundances in parentheses)

Ion assignment	Compound-(*m/z*)		
	5	**6**	**7**
$[M]^{+\cdot}$	456[a]	630 (<1)	514 (<1)
$[M-{}^{\cdot}CH_3]^+$		615 (<1)	
$[M-CH_3O^{\cdot}]^+$		599 (<1)	
$[M-CH_3OH]^{+\cdot}$	424 (20)		482 (10)
$[M-(2XCH_3OH)]^{+\cdot}$	392 (31)		450 (5)
$[M-{}^{\cdot}C_5H_{11}]^+$	383 (2)	559 (<1)	
[M—TMSOH]$^+$		540 (2)	
$[M-(CH_3OH + {}^{\cdot}C_5H_{11})]^+$	353 (40)	411 (25)	
$[M-(TMSOH + CH_3O^{\cdot})]^+$			509 (2)
$[M-(ZO-CH-C_5H_{11} + CH_3OH)]^+$	309 (70)		367 (58)
$[M-(YO-CH-CH=CH-CH(OZ)-C_5H_{11})]^+$ (*a*)	185 (100)	301 (42)	243 (100)
$[M-(HC=CH-CH(OZ)-C_5H_{11})]^+$	141[b] (7)	199 (4)	141[b] (6)
$[a-CH_3OH]^+$	153 (35)		211 (30)
[*a*—TMSOH]$^+$		211 (100)	153 (84)
$[M-(XO-CH-CH_2-COOCH_3)]^+$	117 (38)	175 (14)	117 (23)
$[ZO-CH-C_5H_{11}]^+$	115 (43)	173 (12)	
$[M-(2XTMSOH)]^{+\cdot}$		450 (12)	
$[{}^{\cdot}CH_2-CH=CH-(CH_2)_3-COOCH_3]^+$	141[c] (7)	141 (3)	141[c] (6)

[a]Not observed.
[b]The *m/z* 141 fragment is also contributed by $[{}^{\cdot}CH_2-CH=CH-(CH_2)_3]^+$.
[c]The *m/z* 141 fragment is also contributed by $[HC=CH-CH(OZ)-C_5H_{11}]^+$.

$[M-{}^{\cdot}OCH_3]^+$, $[M-CH_3OH]^{+\cdot}$, $[M-({}^{\cdot}OCH_3 + CH_3OH)]^+$, $[M-\{{}^{\cdot}CH_2-(CH_2)_3-CH_3\}]^+$ and [M—TMSOH]$^{+\cdot}$. An abundant *m/z* 367 ion indicates cleavage at C-14/C-15 and presence of a methyl ether function at C-15. The ion at *m/z* 243 (C-12/20 fragment from C-8/C-12 cleavage) provides the base peak which then eliminates the elements of trimethylsilanol or of methanol to give fragments at *m/z* 153 and 211, respectively. In the spectrum of the dimethyl ether derivative, a weak molecular ion was observed at *m/z* 424, the basic peak was detected at *m/z* 185 (C-12/20 chain), and the second most abundant ion at *m/z* 309, $[M-(C\text{-}15/20)^{\cdot}]^+$. (The latter ion is analogous to the *m/z* 367 fragment in the spectrum of the alkenic monomethyl ether derivative discussed above). The usual $[M-{}^{\cdot}OCH_3]^+$, $[M-CH_3OH]^{+\cdot}$, $[M-({}^{\cdot}OCH_3 + CH_3OH)]^+$, $[M-(C\text{-}16/20)^{\cdot}]^+$ and $[M-\{CH_3OH + (C\text{-}16/20)^{\cdot}\}]^+$ fragments are also observed along with $[CH_3-(CH_2)_4-CH-OCH_3]^+$ (*m/z* 115). The ion of *m/z* 185 (base peak) eliminates the elements of methanol with formation of a *m/z* 153 fragment.

2.3. *n*-Butylboronate esters

When the reaction mixture was treated with *n*-butylboronic acid [6] immediately after the second methylation with diazomethane (Scheme 1), molecules with still underivatized hydroxyl groups at C-9 and C-12, and only those, were converted to the 9,12-*n*-butylboronate ester derivatives. BSTFA/piperidine (1:1, v/v), unlike BSTFA/pyridine (1:1, v/v) which is normally used for trimethylsilylation, readily splits off the *n*-butylboronic acid moiety with formation of the corresponding TMS ether derivatives at C-9 and C-12. The spectra of two *n*-butylboronate esters are shown in Figs. 4 and 5.

2.4. Experiments with ^{13}C-labeled analogs of 1 and 3

When the second methylation (Scheme 1) was conducted with $^{13}CH_2N_2$, $^{13}C_2$ analogs of **1** and **3** were isolated. In the analog of **1**, (**1**-$^{13}C_2$), the label is on the ester function at C-11 and on the methyl ether function at C-12. In the analog of **3** (**3**-$^{13}C_2$) the label is on the C-11 methyl ester

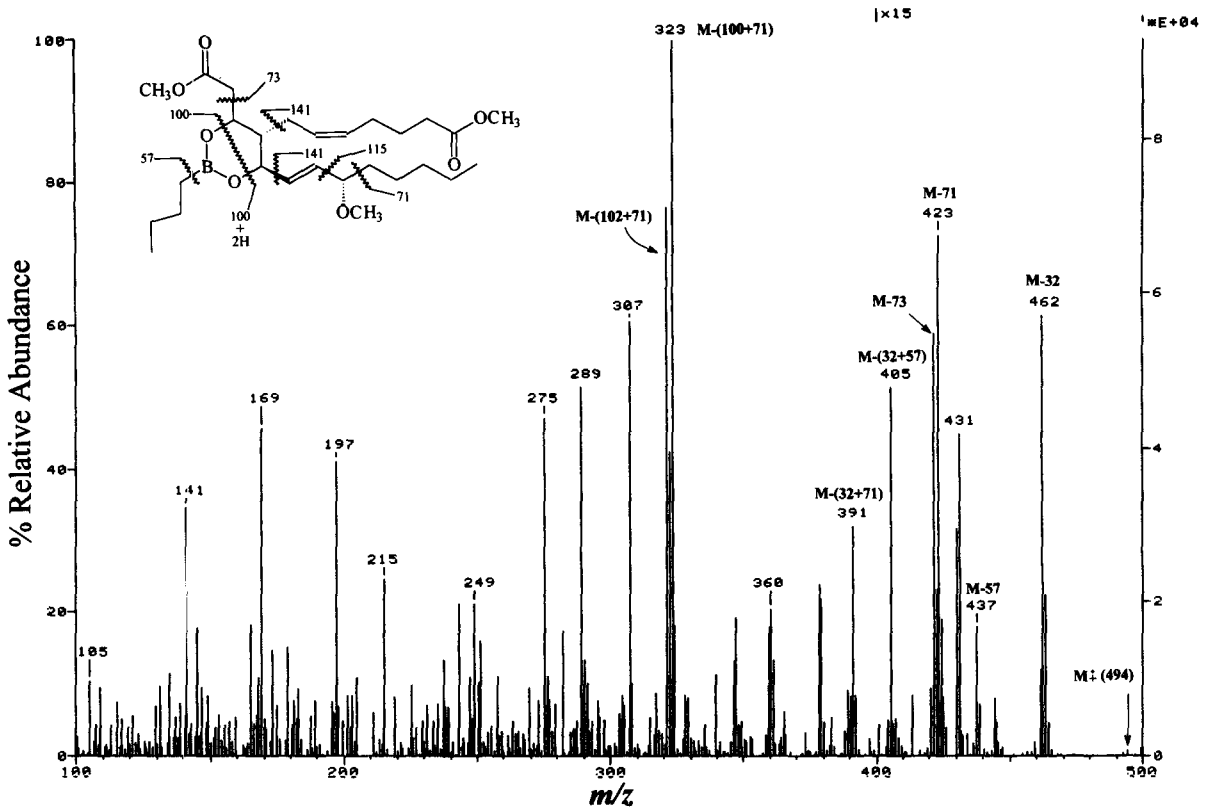


Fig. 4. EI mass spectrum of the 9,12-*n*-butylboronate ester analog of **2**.

moiety and on the OCH_3 at C-9. In both spectra, the molecular ion is shifted 2 u to *m/z* 574. In the spectrum of **1**-$^{13}C_2$, the fragments of *m/z* 243 and *m/z* 175 are shifted to *m/z* 244 and 176, respectively, while the *m/z* 173 ion remains unchanged. Furthermore, ions at *m/z* 526 $[M-(^{13}CH_3OH + {}^{\cdot}CH_3)]^+$ and *m/z* 470 $[M-\{{}^{\cdot}(CH_2)_4-CH_3 + {}^{13}CH_3OH\}]^+$ are observed. In the spectrum of **3**-$^{13}C_2$, the ion at *m/z* 117 (C-8/C-9 cleavage) is shifted to 119, while those at *m/z* 301 and 211 (Table 1) remain unchanged. Fragments arising from the elimination of TMSOH, $(C_5H_{11} + {}^{13}CH_3OH)$ and (TMSOH $+ {}^{13}CH_3OH$) are also observed. The above observations are consistent with the indicated site of the label and with the assignments shown in Table 1.

3. Discussion and conclusions

3.1. Chemistry

Formation of all the products identified can be rationalized on the basis of the following inferences: (1) the alcoholic hydroxyl at C-9 can be partially methylated with diazomethane when 11-$DTXB_2$ is in the open form; (2) the C-9 hydroxyl group is converted to the TMS derivative only with difficulty when 11-$DTXB_2$ is in the lactone form: BSTFA/piperidine, instead of the standard BSTFA/pyridine, must be used to ensure acceptable yields; (3) the allylic hydroxyl groups at C-12 and C-15 can both be partially methylated with diazomethane when the substrate is in the open form. The underlying mechanisms of these anomalous behaviors are not readily apparent. As

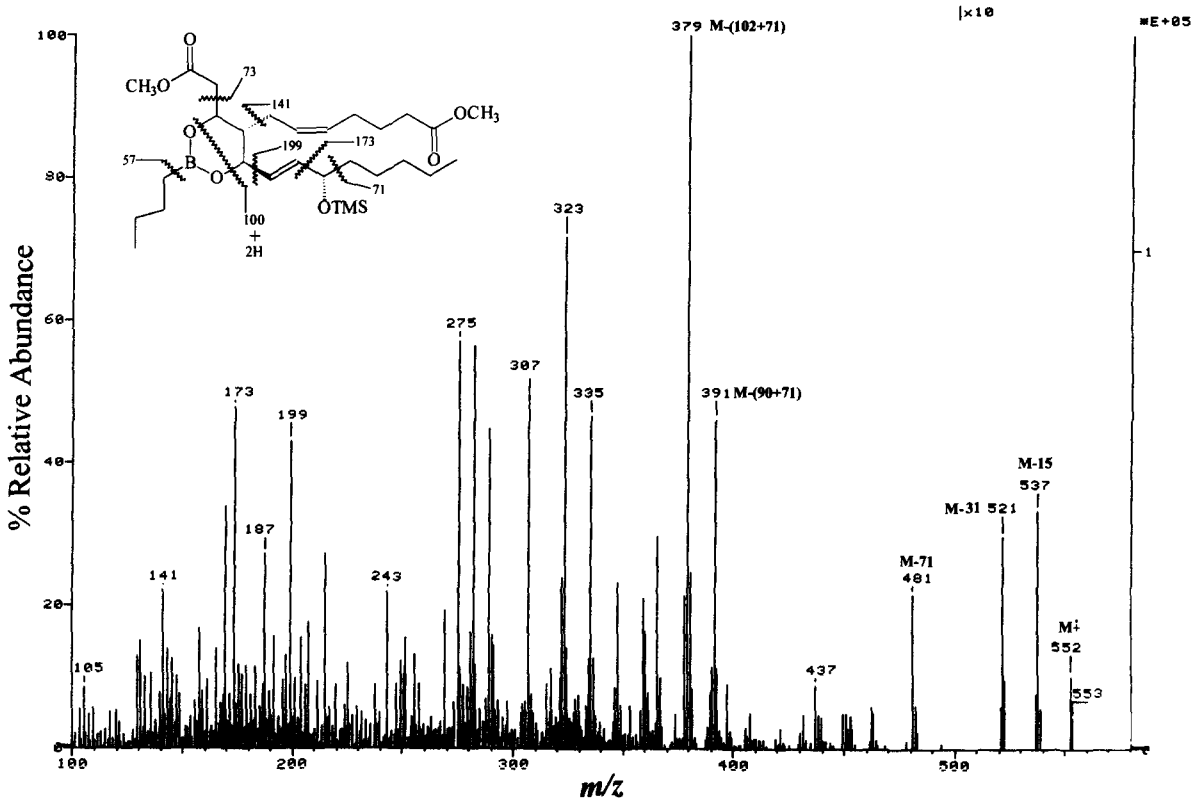


Fig. 5. EI mass spectrum of the 9,12-*n*-butylboronate ester analog of **6**.

for the methylation of the hydroxyl groups at C-9, C-12 and C-15 by diazomethane, perhaps the borate buffer could somehow be acting as a catalyst. Fig. 6 shows a typical total ion current chromatogram. The relative proportions of the products found at the end of the chemical treatment (Scheme 1) vary from experiment to experiment (as estimated by chromatographic peak area measurements) and seem unrelated to the length of time of exposure of the substrate to diazomethane. Formation of similar products, although in different proportions, was also observed when the lactone ring opening was carried out with a mixture of sodium carbonate and sodium bicarbonate (pH 10.5). When 11-dehydrothromboxane B_2 (Fig. 1) is treated with diazomethane according to standard procedure, only the carboxyl group at C-1 undergoes derivatization.

11 - Dehydrothromboxane B_2
↓
Methylation at C-1
↓
Ring opening
↓
Relactonization
↓
Extraction
↓
Remethylation
↓
Trimethylsilylation

Scheme 1.

3.2. Mass spectrometry of 1–7

The most characteristic ions in the spectra of **1–7** originate from cleavage at C-8/C-12 with charge retention by both fragments. The ions thus produced indicate the type of derivatization at C-12 and C-15: a fragment at *m/z* 301 indicates bis-TMS derivatization, while a usually abundant

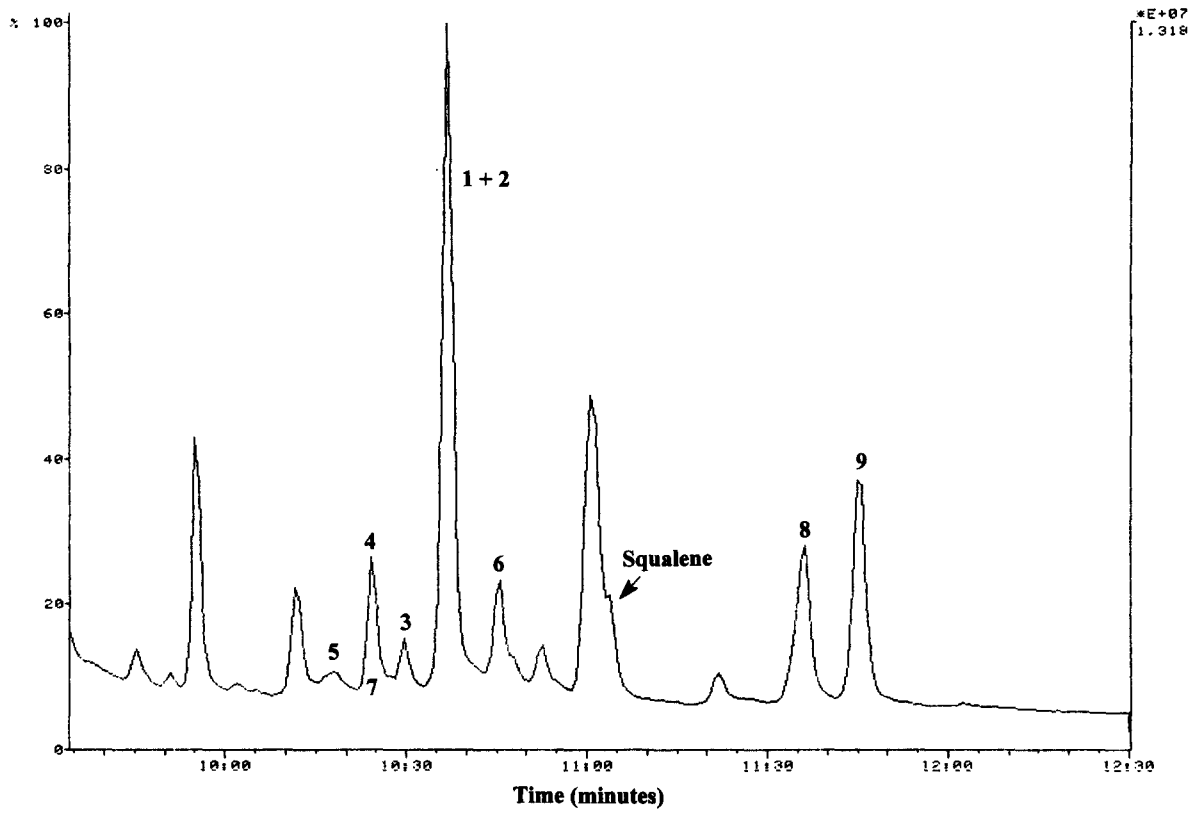


Fig. 6. Typical total ion current chromatogram. Bold numbers indicate the compound(s) associated with a given peak (see Figs. 2 and 3).

ion at m/z 243 indicates a mixed (methyl-TMS) ether derivative. The primary species of m/z 301 in the spectra of **3** and **6** then produce ions at m/z 211 upon elimination of TMSOH. The m/z 243 fragments in the spectra of **1**, **2** and **7** lose the elements of methanol to produce ions also of m/z 211. The m/z 185 species in the spectrum of **4** and **5** produce fragments of m/z 153 upon elimination of methanol. Also, generally observed in all spectra, is the elimination from the molecular ions of neutral or radical fragments such as $^{\cdot}CH_3$ (15 u), $CH_3O^{\cdot}$ (31 u), CH_3OH (32 u), TMSOH (90 u), $^{\cdot}C_5H_{11}$ (71 u) and $^{\cdot}CH_2—CH{=}CH—(CH_2)_3—COOCH_3$ (141 u).

The molecular ions are invariably weak and are not detected when the molecule contains more than one methyl ether function. The $[M—32]^{+\cdot}$ fragment, however, is always present and it cannot be mistaken for a molecular ion because it is always accompanied by the $[M—31]^+$ fragment which is much more abundant than the isotope satellite of $[M—32]^+$ would be. Finally, fragment ions $[H_3C—(CH_2)_4—CH—OTMS]^+$ (m/z 173), $[H_3C—(CH_2)_4—CH—OCH_3]^+$ (m/z 115), $[H_3C—O—CO—CH_2—CH—OTMS]^+$ (m/z 175) and $[H_3C—O—CO—CH_2—CH—OCH_3]^+$ (m/z 117) are usually observed in the spectra of the appropriate derivatives.

3.3. Practical considerations

The anomalous behaviors of 11-DTXB_2 described in this paper can interfere with assay developments and must be taken into consideration when chemical manipulations involving the hydroxyls at C-9, C-12 and C-15 are contemplated. The formation of several by-products during the execution of Scheme 1, and during the conduct of our assay for 11-dehydrothromboxane B_2 [4] as well, can be avoided by ensuring quantitative closure of the lactone ring upon acidifica-

tion before proceeding to the next steps. This can be reliably accomplished, at least in our hands, by allowing the reaction mixture to stand at room temperature for a full hour at pH 3. Moreover, as indicated above, trimethylsilylation of the hydroxyl group at C-9 with good yield requires the use of BSTFA in piperidine (rather than in pyridine) when the lactone ring is closed. The anomalies observed in 11-DTXB_2 might be exhibited by other thromboxane metabolites. This is almost certainly the case with 11-dehydro-2,3-dinor-thromboxane B_2 and possibly with 2,3-dinor-thromboxane B_2 and B_1 [7].

4. Experimental

4.1. Materials

11-Dehydrothromboxane B_2 was purchased from Cayman Chemical Company (Ann Arbor, MI), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce Chemical Company (Rockford, IL) and *n*-butylboronic acid from Applied Science Laboratories (State College, PA). Diazomethane was prepared by the action of KOH on *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Company, Milwaukee, WI) and codistilled with diethyl ether. It was stored at −25°C in diethyl ether. Solvents were analytical grade and distilled in glass. All analytical operations were done in silanized glassware.

4.2. Instrumental

Gas chromatography was performed on a Varian 3400 instrument operated in the splitless mode with a 22 m × 0.25 mm DB-1 (J. & W. Scientific, Inc., Rancho Cordova, CA) capillary column. The He flow was 28 cm/s and the injector temperature was 250°C. The oven was kept at 100°C for 1 min after injection, then it was heated to 300°C at the rate of 27°C/min. The gas chromatograph was interfaced to a Finnigan-MAT TSQ-70B triple stage mass spectrometer operated in the EI mode. The interface temperature was 300°C and the ion source temperature was 150°C.

4.3. Preparation of derivatives

The series of derivatizations shown in Scheme 1 were performed on a 50 μg scale. Methylation was carried out by treating the substrate with 3 ml of ethereal diazomethane and three drops of methanol (to ensure complete dissolution). After 15 min at ambient temperature, the solvents were evaporated under dry N_2, and the residue was treated with two drops of methanol and 1 ml of 50 mM borate buffer (pH 10.5) for 1 h at room temperature to effect opening of the lactone ring. The solution was then treated with dilute HCl (to attain relactonization) and extracted with ethyl acetate/hexane (1:1, v/v) immediately upon reaching pH 3. After evaporation of the solvents under dry N_2, the residue was treated again with diazomethane and finally with BSTFA in pyridine (1:1, v/v) at 42°C for 15 min. After evaporation, the residue was dissolved and stored, pending GC-MS analysis, in 2,2,4-trimethylpentane containing 2% BSTFA/pyridine (1:1, v/v).

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